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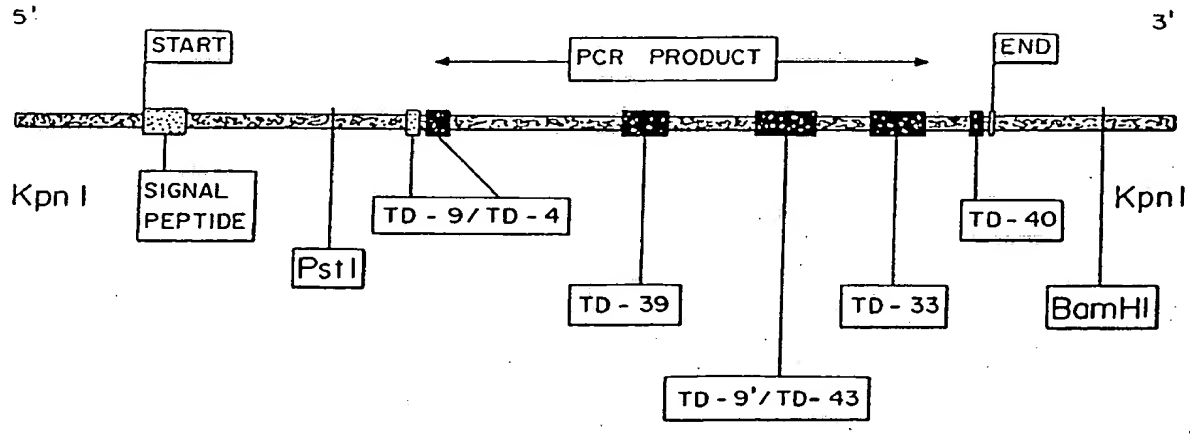

(57) Abstract

The cloning of the heparinase gene from *Flavobacterium Heparinum* using the polymerase chain reaction is described. The Open Reading Frame (ORF) corresponded to 1152 base pairs encoding a precursor protein of MW 43,800 daltons. The amino acid sequence reveals a 20-residue leader peptide. The gene was expressed in two expression systems in *E. Coli.*

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HEPARINASE GENE FROM *FLAVOBACTERIUM HEPARINUM*

Background of the Invention

This invention is generally in the area of heparinases and is specifically directed to the gene encoding heparinase I, expressed in Flavobacterium heparinum.

The United States government has rights in this invention by virtue of grant number 25810 from the National Institutes of Health.

Heparin is an anticoagulant that activates serine protease inhibitors (serpins), which play a key role in the blood clotting cascade, as described by Damus et al., *Nature* 246:355-357 (1973). According to Lindahl et al., *Trends Biochem. Sci.* 11:221-225 (1986), heparin is the most acidic natural polymer known to date. It consists of a major 1,4-linked disaccharide repeating unit of D-uronic acid 1,4-ß-D-glucosamine, and has an average of four negative charges (three sulfate groups and one carboxylate group) per monosaccharide unit. Heparin is both polydisperse, having an average molecular weight between 3,000 and 45,000 daltons, and heterogenous due to partial epimerization of D-glucuronic acid to L-iduronic acid and incomplete N- and O- sulfation, as reported by Kusche et al., *Proc. Natl. Acad. Sci.*, 77:6551-6555 (1980) and Comper, *Polymer Monograph* 7, 1981.

In addition, proteoglycans like heparin have a wide range of biological influences, including in blood chemistry, growth factor interaction and wound healing, interaction with basic structural proteins in the extracellular matrix and in cellular mediated immune responses. The basic nature of protein/peptide heparin/complex carbohydrate interaction is important. Although heparin seems fairly heterogenous, it is now quite clear that different heparin fractions exhibit distinct and unique properties indicating some

compositional and possibly structural specificity for its biological role, as reviewed by Cardin, A. D. and H. J. R. Weintraub, *Arteriosclerosis* 9, 21-32 (1989).

Heparinase, also referred to as heparin lyase, is the only known enzyme capable of degrading heparin that has been extensively characterized. It has been designated EC 4.2.2.7 by the Enzyme Commission. According to Galliher, et al., *Eur. J. Appl. Microbiol.* 15:252 (1982), the enzyme is a polysaccharide lyase found in the periplasmic space of *Flavobacterium heparinum*, a Gram-negative soil isolate. *F. heparinum* utilizes heparin as its sole source of carbon and nitrogen, as described by Hoving and Linker, *J. Biol. Chem.* 245:6170 (1970). Heparinase is the initial enzyme of heparin catabolism. Although constitutively expressed in low amounts, Galliher, et al., *App. Environ. Microbiol.* 41:360 (1981), have discovered that enzyme expression is induced by heparin and reversibly repressed by sulfate in the medium. Lindhardt, et al., *Appl. Biochem. Biotechnol.* 9:41 (1984), have shown that heparinase is inhibited by other polyanionic polysaccharides.

Heparinase has been purified by standard chromatographic techniques and its enzymatic properties characterized extensively, as described by scientists including Yang, et al., *J. Biol. Chem.* 260:1849 (1985). The enzyme is a 44,000 dalton monomeric protein with a pI of approximately 9.

Heparinase acts as an eliminase, leaving behind an unsaturated double bond at the non-reducing end group. This double bond is exploited in an assay for heparinase activity by the absorbance of the unsaturated product at 232 nm. The enzyme is marginally tolerant to salts and is very specific for heparin, having a k_d of 30 nM. Heparinase has an

activation energy of 4.5 kcal/mol, a km of 8 x 10-6 and a Vmax of 4 x 10-7 M/min.

Heparin is often used in surgery to prevent blood clotting and to increase the compatibility of extracorporeal devices such 30 as heart-lung and kidney dialysis machines. The enzymatic degradation of heparin by heparinase is sufficient to eliminate the anticoagulation properties of heparin in surgery. As described by Langer, et al. in *Biomaterials: Inter-facial Phenomenon and Applications, Adv. in Chem. Symposium Series*, Chap. 13, pp. 493-509 (1982), this property has led to the use of heparinase as an immobilized bioreactor in conjunction with heart-lung or kidney dialysis machines to deheparinize blood. Commercial application of the heparinase bioreactor is pending clinical trials.

A principal problem in the use of the heparinase bioreactor is the availability of sufficient amounts of pure heparinase to be immobilized onto a surface. This is primarily because the amount of heparinase constitutively expressed in *F. heparinum* is very low. Inducing expression of heparinase in *F. heparinum* with heparin is very expensive due to the amounts of heparin needed and the size of the fermentation to produce reasonable amounts of heparinase for any practical applications.

Cloning and expression of the heparinase gene is important in several ways. First, the only enzyme cloned and characterized to date which acts to depolymerise proteoglycans is heparinase. Second, heparin is the only anticoagulant commonly used in surgery so deheparinizing blood is an important medical problem. Moreover, heparinase catalyzed degradation of heparin into lower molecular weight heparin molecules can be used to yield products with specific anticoagulant activity, as discussed by

Rosenfeld and Danishefsky, *Biochem. J.* 237:639-646 (1986).

Designing recombinant heparinases with altered activitie(s) would be interesting academically, as well as commercially. For example, heparinase can be used to deheparinize blood because the enzyme cleaves right at the AT-III binding oligomer. On the other hand, by further understanding the mechanism of the enzyme binding and depolymerizing heparin, recombinant heparinases with altered specificity could be designed, i.e. an AT-III binding heparin fragment not cleaved by the recombinant enzyme. This would be a very useful way of generating an AT-III binding heparin oligosaccharide, which currently is not available in large amounts, for use as an anticoagulant. Producing heparinases which could help and or improve in the enzyme purification or immobilization would also be quite valuable. For example, a tag (a particular peptide sequence) could be added at a region which does not alter the activity of the enzyme but makes the immobilization chemistry very efficient. This would help in improving enzyme loading onto the immobilization matrix.

It is therefore an object of the present invention to provide the gene encoding heparinase and a system for expression to facilitate the production of large amounts of heparinase.

It is another object of the present invention to provide methods and means for modifying the gene to produce recombinant heparinases having altered specificity and other desirable properties.

It is another object of the present invention to provide pure heparinase for use in the area of cytokine-proteoglycan interactions, as a tool or diagnostic as exemplified by fibroblast growth factor - heparin interactions.

Summary of the Invention

The cloning of the heparinase gene from *Flavobacterium Heparinum* using the polymerase chain reaction is described. Two degenerate oligonucleotides, based on amino acid sequence derived from tryptic peptides of purified heparinase were used in the PCR with *Flavobacterium* genomic DNA as the template to generate a 600 base pairs probe. This probe was used to screen a pUC 18 *Flavobacterium* genomic library. The Open Reading Frame (ORF) corresponded to 1152 base pairs encoding a precursor protein of MW 43,800 daltons. Eleven different tryptic peptides (approximately 48% of the total amino acids) mapped into the ORF. The amino acid sequence reveals a 20-residue leader peptide.

Heparinase can be expressed from the gene. Additionally, the gene can be modified to produce heparinase with altered enzymatic activity, specificity, or binding properties. The sequence can also be used as a probe in the isolation of genes encoding other related enzymes.

Brief Description of the Drawings

Figure 1 is a schematic representation of the PCR products Y1:C and D:C which are 600 and 160 basepairs, respectively. The 600 basepair PCR product was used as a template with D and C as primers to generate the 160 basepair D:C product.

Figure 2 is the restriction map of the genomic DNA pUC 18 plasmid, pRS.HEP51, having an insert containing the heparinase gene. The plasmid is 5631 bases long and has approximately 2300 bases of insert. The heparinase gene is in the *Kpn I-KpnI* fragment.

Figure 3 is a *KpnI-KpnI* fragment map showing the heparinase gene structure with the different tryptic peptides mapping into the open reading frame. Six

different peptides mapped into the heparinase gene translation region.

Detailed Description of the Invention

The gene encoding heparinase in *F. heparinum* has been cloned. The nucleotide and amino acid sequences are shown below:

The following sequence (Sequence No. 1, base pairs 1 to 172, inclusive) encodes a leader peptide:

CCTTT TGGGA GCAAA GGCAG AACCA TCTCC GAACA AAGGC AGAAC
CAGCC TGTAA ACAGA CAGCA ATTCA TCCGC TTTCA ACCAA AGTGA
AAGCA TTTAA TACAA TACCA GAATG TCGCA TTTCC CTTTC AGCGT
ACTTT TTGGG TAAAT AACCA ATAAA AACTA AAGAC GG

The following sequence (Sequence No. 1, base pairs 173 to 1379, inclusive) encodes the heparinase:

ATG AAA AAA CAA ATT CTA TAT CTG ATT GTA CTT CAG CAA
CTG TTC CTC TGT TCG GCT TAC GCC CAG CAA AAA AAA TCC
GGT AAC ATC CCT TAC CGG GTA AAT GTG CAG GCC GAC AGT
GCT AAG CAG AAG GCG ATT ATT GAC AAC AAA TGG GTG GCA
GTA GGC ATC AAT AAA CCT TAT GCA TTA CAA TAT GAC GAT
AAA CTG CGC TTT AAT GGA AAA CCA TCC TAT CGC TTT GAG
CTT AAA GCC GAA GAC AAT TCG CTT GAA GGT TAT GCT GCA
GGA GAA ACA AAG GGC CGT ACA GAA TTG TCG TAC AGC TAT
GCA ACC ACC AAT GAT TTT AAG AAA TTT CCC CCA AGC GTA
TAC CAA AAT GCG CAA AAG CTA AAA ACC GTT TAT CAT TAC
GGC AAA GGG ATT TGT GAA CAG GGG AGC TCC CGC AGC TAT
ACC TTT TCA GTG TAC ATA CCC TCC TCC TTC CCC GAC AAT
GCG ACT ACT ATT TTT GCC CAA TGG CAT GGT GCA CCC AGC
AGA ACG CTT GTA GCT ACA CCA GAG GGA GAA ATT AAA ACA
CTG AGC ATA GAA GAG TTT TTG GCC TTA TAC GAC CGC ATG
ATC TTC AAA AAA AAT ATC GCC CAT GAT AAA GTT GAA AAA
AAA GAT AAG GAC GGA AAA ATT ACT TAT GTA GCC GGA AAG
CCA AAT GGC TGG AAG GTA GAA CAA GGT GGT TAT CCC ACG
CTG GCC TTT GGT TTT TCT AAA GGG TAT TTT TAC ATC AAG
GCA AAC TCC GAC CGG CAG TGG CTT ACC GAC AAA GCC GAC
CGT AAC AAT GCC AAT CCC GAG AAT AGT GAA GTA ATG AAG
CCC TAT TCC TCG GAA TAC AAA ACT TCA ACC ATT GCC TAT

AAA ATG CCC TTT GCC CAG TTC CCT AAA GAT TGC TGG ATT
ACT TTT GAT GTC GCC ATA GAC TGG ACG AAA TAT GGA AAA
GAG GCC AAT ACA ATT TTG AAA CCC GGT AAG CTG GAT GTG
ATG ATG ACT TAT ACC AAG AAT AAG AAA CCA CAA AAA GCG
CAT ATC GTA AAC CAG CAG GAA ATC CTG ATC GGA CGT AAC
GAT GAC GAT GGC TAT TAC TTC AAA TTT GGA ATT TAC AGG
GTC GGT AAC AGC ACG GTC CCG GTT ACT TAT AAC CTG AGC
GGG TAC AGC GAA ACT GCC AGA TAG (stop codon)

The following is the amino acid sequence (Sequence No. 2) of heparinase:

Met Lys Lys Gln Ile Leu Tyr Leu Ile Val Leu Gln Gln
Leu Phe Leu Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser
Gly Asn Ile Pro Tyr Arg Val Asn Val Gln Ala Asp Ser
Ala Lys Gln Lys Ala Ile Ile Asp Asn Lys Trp Val Ala
Val Gly Ile Asn Lys Pro Tyr Ala Leu Gln Tyr Asp Asp
Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr Arg Phe Glu
Leu Lys Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala Ala
Gly Glu Thr Lys Gly Arg Thr Glu Leu Ser Tyr Ser Tyr
Ala Thr Thr Asn Asp Phe Lys Lys Phe Pro Pro Ser Val
Tyr Gln Asn Ala Gln Lys Leu Lys Thr Val Tyr His Tyr
Gly Lys Gly Ile Cys Glu Gln Gly Ser Ser Arg Ser Tyr
Thr Phe Ser Val Tyr Ile Pro Ser Ser Phe Pro Asp Asn
Ala Thr Thr Ile Phe Ala Gln Trp His Gly Ala Pro Ser
Arg Thr Leu Val Ala Thr Pro Glu Gly Glu Ile Lys Thr
Leu Ser Ile Glu Glu Phe Leu Ala Leu Tyr Asp Arg Met
Ile Phe Lys Lys Asn Ile Ala His Asp Lys Val Glu Lys
Lys Asp Lys Asp Gly Lys Ile Thr Tyr Val Ala Gly Lys
Pro Asn Gly Trp Lys Val Glu Gln Gly Gly Tyr Pro Thr
Leu Ala Phe Gly Phe Ser Lys Gly Tyr Phe Tyr Ile Lys
Ala Asn Ser Asp Arg Gln Trp Leu Thr Asp Lys Ala Asp
Arg Asn Asn Ala Asn Pro Glu Asn Ser Glu Val Met Lys
Pro Tyr Ser Ser Glu Tyr Lys Thr Ser Thr Ile Ala Tyr
Lys Met Pro Phe Ala Gln Phe Pro Lys Asp Cys Trp Ile
Thr Phe Asp Val Ala Ile Asp Trp Thr Lys Tyr Gly Lys
Glu Ala Asn Thr Ile Leu Lys Pro Gly Lys Leu Asp Val
Met Met Thr Tyr Thr Lys Asn Lys Lys Pro Gln Lys Ala

His Ile Val Asn Gln Gln Glu Ile Leu Ile Gly Arg Asn Asp Asp Asp Gly Tyr Tyr Phe Lys Phe Gly Ile Tyr Arg Val Gly Asn Ser Thr Val Pro Val Thr Tyr Asn Leu Ser Gly Tyr Ser Glu Thr Ala Arg.

Example 1: **Isolation and analysis of CDNA encoding heparinase in *F. heparinum*.**

Because preliminary cloning attempts by others utilizing 1) antibody screening, 2) screening for functionally active heparinase in *E.coli* and 3) screening for the heparinase gene using probes derived from protein sequences regenerated by cyanogen bromine (CNBr) chemical digest were unsuccessful, the polymerase chain reaction was used to clone the heparinase gene. The reverse phase purified heparinase was reduced, alkylated and digested with trypsin to obtain approximately 60 peptide peaks which were separated and collected by reverse phase HPLC monitored at 210 nm and at 277 nn (for tyrosine and tryptophan), as described below.

Tryptic Digest and Protein Sequence Analyses

Heparinase was purified as described by Dietrich, et al., *J. Biol. Chem.* 248:6408 (1973), Otatani et al., *Carbohyd. Res.* 88:291 (1981), and Yang et al., *J. Biol. Chem.* 260:1849 (1985), which are incorporated by reference herein. A final purification step was carried out by High Performance Liquid Chromatography (HPLC) using a reverse phase column that exploits the hydrophobic residues of the protein. A nanomole (approximately 45 μg) of the purified enzyme was denatured in 50 μl of an 8 M urea, 0.4 M ammonium carbonate solution, reduced with 5 mM dithiothreitol (DTT) at 50°C, cooled to room temperature, and alkylated with 10 mM iodoacetamide for 15 minutes in the dark. The total reaction volume was 200 μl. To this reaction mixture, 1/25th w/w of trypsin was added and digestion carried out at 37°C

for 24 hour. The reaction was terminated by heating the sample at 65°C for 2 minutes. The digest was separated by reverse phase HPLC using a gradient of 0 to 80% acetonitrile. The tryptic peptides were monitored at 210 and 277 nm.

The tryptic peaks were collected in Eppendorff tubes. Based on the homogeneity of the peptide peak, eight different peaks were sequenced using an Applied Biosystems sequencer, model 477, with an on-line model 120 PTH amino acid analyzer located in the Biopolymers lab, Center for Cancer Research, MIT. The sequences are set forth in Table I below. The designation (K,R) is used in Table I to indicate that trypsin cuts at either lysine or arginine residues. The asterisks in Table I represent amino acids that could not be determined. The peptide designated td Lx is the longest peptide sequenced having 38 residues. Native heparinase was also sequenced to determine the N-terminus amino acids.

Table I: **Sequences of Tryptic Peptides of Heparinase**

Peptide	Amino Acid Sequence
td 04	(K, R) G I C E Q G S S R
td 09	(K, R) T V Y H Y G K
td 09'	(K, R) T S T I A Y K
td 21	(K, R) F G I Y R
td 33	(K, R) A D I V N Q Q E I L I G R D D * G Y Y F K
td 39	(K, R) I T Y V A G K P N G N K V E Q G G Y P T L A F *
td 43	(K, R) M P F A Q F P K D C W I T F D V A I D * T K
td 40	(K, R) N L S G Y S E T A R
tdm4	K N I A H D K V E K K
td 72	K T L S I E E F L A L Y D R
td Lx	R S Y T F S V Y I P S S F P D N A T T I F A W H G A P S R T L V T P E I K

Three sets of primers were designed and synthesized, as shown in Table II. Primers were synthesized with an Applied Biosystems sequencer, model 477, with an on-line model 120 PTH amino acid analyzer located in the Biopolymers lab, Center for Cancer Research, MIT. These primer sets were used in the PCR amplification system for cloning the heparinase gene. The symbol "I" represents the nucleotide inosine. The amino acids of each peptide, depicted in boldface type, represent the residues chosen for the primer design. Two different sets of primers were constructed for tryptic peptide 33 to reduce the degree of inosine substitution at the 3′ end of the primer.

Table II: Heparinase Primer Design

Peptide: td 04

Amino Acid Sequence:

K G I C E Q G S S R

primers:

y1 5′- AAA GGI AT(T/C/A) TG(T/C) GA(A/G) CA(A/G) GG -3′

y2 5′- CC (C/T)TG (C/T)TC (G/A)CA (T/G/A)AT ICC TTT -3′

Peptide: td 43

Amino Acid Sequence:

(K, R) **M P F A Q F P K D E W I T F C V**

A I D * T K

primers:

D 5′- ATG CCI TT(T/C) GCI CA(A/G) TT(T/C) CCI AA(A/G) GA(T/C) GA -3′

E 3′- TAC GGI AA(A/G) CGI GT(T/C) AA(A/G) GGI TT(T/C) CT(A/G) CT -5′

Peptide: td 33

Amino Acid Sequence:

(K, R) A D **I V N Q Q E I L I G** R D D * G Y Y F K A

primers:

A 5′- ATI AA(T/C) CA(A/G) GA(A/G)ATI (C/T)TI AT(T/C/A) GG -3′

B 5′- CCIATIA(G/A) IAT (T/C)TC (T/C)TG (T/C)TG (A/G)TT ICA (A/C)AT

C 5′- CCIATIA(G/A) IAT (T/C)TC (T/CTG (T/C)TG (A/G)TT ICA (T/G)AT -31

Of the six RHPLC peaks initially sequenced (Table I), three were chosen for primer design. Three sets of primers were designed (Table II). The PCR product of the combination the primers td43 and td33 was about 150 base pairs in length. The combination of td4 and td33 primers were about 600 base pairs. Primer td43 was 5′ to primer td33 and primer td4 was 5′ to td43 primer. Using the PCR product of td4 and td33 as a template and td43 and td4 as primers the predicted 150 base pair product was obtained confirming that td43 was between td4 and td33.

The 600 basepair product shown in Figure 1 represents about 51% of the approximated total 1170 base pairs for the heparinase gene, assuming 43,000 dalton for heparinase and a 110 dalton average amino acid with a molecular weight corresponding to about 390 amino acids times three which is 1170 bases.

The 600 base pair probe was chosen for screening a pUC 18 library by high stringency colony hybridization. Two positive clones were identified which were carried through for three rounds of colony purification.

Genomic DNA, RNA, and Plasmid Library

The *F. heparinum* genomic DNA was isolated by the A.S.A.P.™ kit (Boehringer Mannheim, Indianapolis, IN) with the following modifications. The DNA was desalted over a Sephadex™ G-50 column (Nick column, Pharmacia, Piscataway, NJ) and concentrated using a Centricon™ P-30 (Amicon Division, Beverly, MA) to a final volume of 100 1. From 1 x 10^9 cells, 105-115 g of DNA typically were obtained. Total cellular mRNA was isolated using the

guanidine thiocyanate procedure set forth in the Promega technical information publication TB 087 12/89, Promega Corp., Madison, WI 53711. A pUC 18 plasmid was obtained from Dr. A.J. Sinskey, of the Department of Biology at the Massachusetts Institute of Technology. The library was constructed using the *F. heparinum* genomic DNA. The genomic DNA was sonicated and modified by adding *Eco*RI linkers and then ligated to the pUC 18 vector. DH5a was transformed with the pUC 18 genomic library.

Amplification of the PCR Product

Amplification of the heparinase tryptic digest primers was carried out in a 25 l reaction volume containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.01% gelatin plus the four deoxyribose nucleotide triphosphates (dNTPs) at 200 M, using 0.5 M primer and 3 l of the genomic DNA as the template, 2.5 units of the Taq polymerase (Cetus Corp., Emeryville, CA) and 25 l of mineral oil. The samples were placed on an automated heating block (DNA thermal cycler, Perkin Elmer Corp., Norwalk, CT) programmed for step cycles of temperatures 92°C (2 minutes), 50°C (1 minute) and 72°C (3 minutes). This cycle was repeated 35 times. The final cycle had a 72°C 10 minute extension. The PCR products were analyzed on a 0.8% agarose gel containing 0.6 μg/ml ethidium bromide. The control reaction was provided by the Cetus kit.

Screening of the Flavobacterium heparinum pUC 18 genomic library

The pUC 18 library was titered to give approximately 1500 colonies to be tested by the probe generated by PCR. Each plate had approximately 100 colonies which were grown directly on nitrocellulose, to an appropriate small size, and then duplicated to be grown further overnight.

The PCR probe was labelled using the Random Hexanucleotide™ kit (RHN) (IBI Biochemicals Ltd.) which is described briefly as follows. One microgram DNA from

the PCR product run was isolated from a low melt agarose gel, denatured by boiling at 95°C for 10 minutes, and then chilled on ice. To the denatured DNA were added 10 mM dNTPs (dATP, dGTP, dCTP, dTTP), random hexanucleotides in the reaction buffer, and 50 μCi of ^{32}PdCTP(3000 Ci/mmole). The reaction was carried with Klenow for 30 minutes at 37°C and terminated using 0.2 M EDTA. Following the labelling reaction, the labelled probe was purified from the free nucleotide by using a Sephadex G-50 column (Nick Column, Pharmacia, Piscataway, NJ). The colonies were screened with the labelled probe using standard colony hybridization procedures as described by Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, incorporated herein by reference.

Two positive clones were isolated and the plasmids tested for their ability to generate the 600 basepair PCR product. Both of the clones tested positive and were further characterized by restriction mapping. Clone pRS Hep 51 is a 2.3 kb insert in pUC 18 (shown in Figure 2) with a *Kpn-Kpn* fragment of about 1.6 kb. This fragment was a positive template for generating a 600 basepair PCR product. The *Kpn*I-*Kpn*I fragment of pRS 51 was subcloned into M13 and sequenced.

DNA Sequencing

DNA sequencing was performed using phage M13 and employing the dideoxyadenosine 5′-alpha-^{35}S-triphosphate and Sequenase (US Biochemical Corp, Cleveland, OH) as described by the manufacturer. The sequence data was obtained using successive nested deletions in M13 using T4 DNA polymerase as per Cyclone I Biosystems (International Biotechnologies Inc., New Haven, CT) or sequenced using synthetic oligonucleotide primers.

The sequence reveals a single, continuous open reading frame (ORF) of 1152 basepairs corresponding to

384 amino acids and a leader sequence of about 21 amino acids. The PCR product spans from 566 to 1216 bases from the start site and corresponds to about 57% of the total gene.

Initially six different tryptic peptides mapped into the ORF. Subsequently, five other peptides were sequenced for structural studies and all of them mapped into the ORF, for a total of about 48% of the total 367 amino acids. There are three cysteines in all, one associated with the signal peptide. The signal peptide is typical of prokaryotic sequences, having a charged N-terminal region, a core hydrophobic region and a cleavage region with a standard Ala.xxx.Ala site for cleavage.

Example 2: Expression of the heparinase gene in *E. coli*.

Two different expression systems were selected for the expression of heparinase in *E. coli*: the Omp A expression system and the pKK hyper-expression system. The plasmid designs for both expression systems are shown in Table III.

30 Omp A expression system

The Omp A expression system secretes the protein of interest into the periplasmic space, as directed by the Omp A signal sequence, described by Ghrayeb, et al., *EMBO J.* 3:2437 (1984), incorporated herein by reference. This system was chosen since heparinase is naturally expressed into the periplasmic space of *F. heparinum*. The plasmid is under the control of the *lac* repressor and is induced by the addition of IPTG (isopropyl-ß-D thiogalactoside) to the medium. The plasmid was inserted in the pIN-III Omp A-3 vector.

The heparinase insert was generated by PCR utilizing the N terminal and the C terminal sequences of heparinase with two appropriate restriction sites suitable for cloning into the *Eco*RI-*Bam*HI sites. Two primers were constructed as shown in Table II. The insert was amplified by 5 cycles of PCR and ligated to

the Omp A pIN vector with the *E. coli* periplasmic leader sequence. DH5α was transformed and expression was induced with 1 mM IPTG for 3-5 hours.

As shown in Table III, the construct of the Omp A expression system results in two extra amino acids at the amino terminal of the heparinase gene, Gly and Ile. The heparinase sequence begins with a Gln.

The pKK expression system

The pKK expression system is used for over-expression of proteins in accordance with the methods of Brosius and Holy, *Proc. Natl. Acad. Sci.*, 81: 6929 (1984) and Jaffe et al. , *Biochem.* 27:1869 (1988), incorporated by reference herein. This system contains a strong *tac* promotor which, in appropriate hosts, is regulated by the *lac* repressor and induced by the addition of IPTG, as in the Omp A system. The plasmid pKK223-3 has a pUC 8 multiple cloning site and a strong rrnB ribosomal terminator immediately following the *tac* promotor. The ribosomal binding site of the plasmid was utilized by cloning the heparinase gene into a *Sma*I site, which is about 12 bases from the start codon ATG. Like the Omp A construction, the heparinase insert is obtained by PCR with *Sma*I and *Hind*III restriction sites at the N and the C terminals of the protein. As shown in Table III, the native heparinase leader sequence was used for over-production into the periplasm.

Periplasmic proteins of *E. coli* were isolated by osmotic shock. Briefly, 1.5 ml of cells were centrifuged after induction and washed with 10 mM Tris pH 7.5. The cells were then suspended in 20% sucrose in 10 mM Tris pH 7.5 and 5 μl of 0.5 M EDTA. After a five minute incubation on ice, the cells were centrifuged and osmotically shocked by adding approximately 150 μl water. The periplasmic extract was used to determine enzyme activity. Heparinase activity was determined by monitoring the wavelength at 232 nm and by the Azure A

methods of Bernstein et al., *Methods of Immunology* 137:515 (1988), incorporated herein by reference.

The periplasmic extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli, *Nature* 227:690 (1974) and stained using Coumassie blue. In addition, a Western blot assay was performed to confirm the presence of heparinase using a heparinase monoclonal antibody. Heparinase was electrophoretically transferred from the SDS-PAGE gel onto nitrocellulose using the method of Gershoni and Palade, *Analytical Biochem.* 131:1 (1983), and then incubated with the monoclonal antibody. This antibody was stained using a secondary antibody conjugated to horseradish peroxidase.

Table III: Design of OmpA and pKK plasmids for expression of Recombinant Heparinase in *E.coli*

Omp A secretion Expression system

N	Gly	Ile	Gln	Lys		Thr	Ala	Arg	End	C
XXX	**GGA**	**ATT**	CAG	AAA	--------	ACT	GCC	AGA	TAG	**GGATCCXXX**
XXX	**CCT**	**TAA**	GTC	TTT	--------	TGA	CTT	ACT	ATC	**CCTAGGXXX**
	*Eco*RI								*Bam* HI	

pKK over-Expression system

N	Met	Lys	Lys			Ala	Arg	End					
XXX	Taa	**CCC**	**GGG**	ATG	AAA	AAA -----	GCC	AGA	TAG	**AAG**	**CTT**	CCG	XXX
XXX	ATT	**GGC**	**CCC**	TAC	TTT	TTT -----	CGG	TCT	ATC	**TTC**	**GAA**	GGC	XXX
		Sma I											*Hind* III

RNA Dot Blot Assay

The total cellular PNA was immobilized onto a Zeta probe™ membrane (Biorad, Richmond, CA) by alkaline RNA denaturation and fixation, and probed using the 600 base PCR product, used in screening for the heparinase gene. The hybridization was carried out with dot blot apparatus in accordance with the method of Thomas, *Proc. Natl. Acad Sci.* 77:5201 (1980). The RNA signal under different growth conditions has been investigated by Galliher, et al., *Eur. J. Appl. Microbiol.* (1982). It was established by those studies that heparinase at the protein level is optimally expressed under low sulphur conditions, which removes the requirement of heparin for induction. Heparinase mRNA signal under low sulphur growth conditions was therefore studied with and without heparin induction.

Both the OmpA and the pKK systems expressed heparinase. The OmpA system did not efficiently transport heparinase to the periplasm. For reasons not known, a large fraction of recombinant heparinase was retained in the cytoplasmic region along with the Omp A signal sequence. At lower temperatures (25°-30°C) of growth, there was some secretion into the periplasmic space.

The pKK overproduction system produced heparinase only in the periplasmic space. The pKK system used the native *F.heparinum* heparinase leader sequence in which there was no problem with the transport of the recombinant protein with a foreign leader sequence. The pKK system expressed heparinase without any aberrant processing, although the expression was again optimal at lower temperatures. The presence of heparinase in the periplasm was confirmed by western blotting and by comparing *in situ* tryptic digest of the recombinant heparinase with that of the native heparinase, in terms

of the peak profiles and some peaks which were isolated and sequenced.

A positive signal was obtained for the isolated *F. heparinum* mRNA using the 600 basepair probe derived from the PCR which has been used for isolating the heparinase gene, confirming that the gene isolated was a *F. heparinum* gene cloned in *E. coli*.

The expressed heparinase appeared to have at least some heparinase activity.

The sequence can be modified to alter specific enzymatic activity or binding specificity or affinity by substitution of one or more amino acids, using site directed mutagenesis or substitution of oligomers into the sequence encoding the heparinase. Methods and materials to accomplish this are known to those skilled in the art. The modified gene is then expressed and the product routinely screened for the altered activity.

Although described with reference to two specific expression systems, other expression systems are well known and commercially available. The heparinase gene can be expressed in these systems, using similar vectors and signal peptides or leader sequences.

Modifications and variations of the present invention will be obvious to those skilled in the art. Such modifications and variations are intended to come within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Massachusetts Institute, of Technology

(ii) TITLE OF INVENTION: The Heparinase Gene from Flavobacterium Heparinum

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Kilpatrick & Cody
(B) STREET: 1100 Peachtree Street, Suite 2800
(C) CITY: Atlanta
(D) STATE: Georgia
(E) COUNTRY: US
(F) ZIP: 30309-4530

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Pabst, Patrea L.
(B) REGISTRATION NUMBER: 31,284
(C) REFERENCE/DOCKET NUMBER: MIT5546

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 404-815-6508
(B) TELEFAX: 404-815-6555

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1379 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Flavobacterium heparinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTTTTGGGA GCAAAGGCAG AACCATCTCC GAACAAAGGC AGAACCAGCC TGTAAACAGA 60

CAGCAATTCA TCCGCTTTCA ACCAAAGTGA AAGCATTTAA TACAATACCA GAATGTCGCA 120

TTTCCCTTTC AGCGTACTTT TTGGGTAAAT AACCAATAAA AACTAAAGAC GGATGAAAAA 180

ACAAATTCTA TATCTGATTG TACTTCAGCA ACTGTTCCTC TGTTCGGCTT ACGCCCAGCA 240

AAAAAAATCC GGTAACATCC CTTACCGGGT AAATGTGCAG GCCGACAGTG CTAAGCAGAA 300

GGCGATTATT GACAACAAAT GGGTGGCAGT AGGCATCAAT AAACCTTATG CATTACAATA 360

TGACGATAAA CTGCGCTTTA ATGGAAAACC ATCCTATCGC TTTGAGCTTA AAGCCGAAGA 420

CAATTCGCTT GAAGGTTATG CTGCAGGAGA AACAAAGGGC CGTACAGAAT TGTCGTACAG 480

CTATGCAACC ACCAATGATT TTAAGAAATT TCCCCCAAGC GTATACCAAA ATGCGCAAAA 540

GCTAAAAACC GTTTATCATT ACGGCAAAGG GATTTGTGAA CAGGGGAGCT CCCGCAGCTA 600

TACCTTTTCA GTGTACATAC CCTCCTCCTT CCCCGACAAT GCGACTACTA TTTTTGCCCA 660

ATGGCATGGT GCACCCAGCA GAACGCTTGT AGCTACACCA GAGGGAGAAA TTAAAACACT 720

GAGCATAGAA GAGTTTTTGG CCTTATACGA CCGCATGATC TTCAAAAAAA ATATCGCCCA 780

TGATAAAGTT GAAAAAAAAG ATAAGGACGG AAAAATTACT TATGTAGCCG GAAAGCCAAA 840

TGGCTGGAAG GTAGAACAAG GTGGTTATCC CACGCTGGCC TTTGGTTTTT CTAAAGGGTA 900

TTTTTACATC AAGGCAAACT CCGACCGGCA GTGGCTTACC GACAAAGCCG ACCGTAACAA 960

TGCCAATCCC GAGAATAGTG AAGTAATGAA GCCCTATTCC TCGGAATACA AAACTTCAAC 1020

CATTGCCTAT AAAATGCCCT TTGCCCAGTT CCCTAAAGAT TGCTGGATTA CTTTTGATGT 1080

CGCCATAGAC TGGACGAAAT ATGGAAAAGA GGCCAATACA ATTTTGAAAC CCGGTAAGCT 1140

GGATGTGATG ATGACTTATA CCAAGAATAA GAAACCACAA AAAGCGCATA TCGTAAACCA 1200

GCAGGAAATC CTGATCGGAC GTAACGATGA CGATGGCTAT TACTTCAAAT TTGGAATTTA 1260

CAGGGTCGGT AACAGCACGG TCCCGGTTAC TTATAACCTG AGCGGGTACA GCGAAACTGC 1320

CAGATAGCAA AAGCCCTAAG CGCATCCGAT AGGGCTTTTC TTATATTTAC AATAAAATT 1379

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 384 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Flavobacterium heparinum

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Lys Lys Gln Ile Leu Tyr Leu Ile Val Leu Gln Gln Leu Phe Leu
1               5                  10                  15

Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser Gly Asn Ile Pro Tyr Arg
            20                  25                  30

Val Asn Val Gln Ala Asp Ser Ala Lys Gln Lys Ala Ile Ile Asp Asn
        35                  40                  45

Lys Trp Val Ala Val Gly Ile Asn Lys Pro Tyr Ala Leu Gln Tyr Asp
    50                  55                  60

Asp Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr Arg Phe Glu Leu Lys
65                  70                  75                  80

Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala Ala Gly Glu Thr Lys Gly
                85                  90                  95
```

```
Arg Thr Glu Leu Ser Tyr Ser Tyr Ala Thr Thr Asn Asp Phe Lys Lys
          100                 105                 110

Phe Pro Pro Ser Val Tyr Gln Asn Ala Gln Lys Leu Lys Thr Val Tyr
      115                 120                 125

His Tyr Gly Lys Gly Ile Cys Glu Gln Gly Ser Ser Arg Ser Tyr Thr
  130                 135                 140

Phe Ser Val Tyr Ile Pro Ser Ser Phe Pro Asp Asn Ala Thr Thr Ile
145                 150                 155                 160

Phe Ala Gln Trp His Gly Ala Pro Ser Arg Thr Leu Val Ala Thr Pro
                165                 170                 175

Glu Gly Glu Ile Lys Thr Leu Ser Ile Glu Glu Phe Leu Ala Leu Tyr
          180                 185                 190

Asp Arg Met Ile Phe Lys Lys Asn Ile Ala His Asp Lys Val Glu Lys
      195                 200                 205

Lys Asp Lys Asp Gly Lys Ile Thr Tyr Val Ala Gly Lys Pro Asn Gly
  210                 215                 220

Trp Lys Val Glu Gln Gly Gly Tyr Pro Thr Leu Ala Phe Gly Phe Ser
225                 230                 235                 240

Lys Gly Tyr Phe Tyr Ile Lys Ala Asn Ser Asp Arg Gln Trp Leu Thr
                245                 250                 255

Asp Lys Ala Asp Arg Asn Asn Ala Asn Pro Glu Asn Ser Glu Val Met
          260                 265                 270

Lys Pro Tyr Ser Ser Glu Tyr Lys Thr Ser Thr Ile Ala Tyr Lys Met
      275                 280                 285
```

Pro Phe Ala Gln Phe Pro Lys Asp Cys Trp Ile Thr Phe Asp Val Ala
290 295 300

Ile Asp Trp Thr Lys Tyr Gly Lys Glu Ala Asn Thr Ile Leu Lys Pro
305 310 315 320

Gly Lys Leu Asp Val Met Met Thr Tyr Thr Lys Asn Lys Lys Pro Gln
325 330 335

Lys Ala His Ile Val Asn Gln Glu Ile Leu Ile Gly Arg Asn Asp
340 345 350

Asp Asp Gly Tyr Tyr Phe Lys Phe Gly Ile Tyr Arg Val Gly Asn Ser
355 360 365

Thr Val Pro Val Thr Tyr Asn Leu Ser Gly Tyr Ser Glu Thr Ala Arg
370 375 380

We claim:

1. An isolated nucleic acid molecule isolated from *Flavobacterium heparinum* encoding heparinase I.

2. The nucleic acid molecule of claim 1 having the nucleotide sequence (Sequence No. 1, base pairs 173 to 1324, inclusive) consisting essentially of:

ATGAAAAA 180

ACAAATTCTA TATCTGATTG TACTTCAGCA ACTGTTCCTC TGTTCGGCTT 230
ACGCCCAGCA AAAAAAATCC GGTAACATCC CTTACCGGGT AAATGTGCAG 280
GCCGACAGTG CTAAGCAGAA GGCGATTATT GACAACAAAT GGGTGGCAGT 330
AGGCATCAAT AAACCTTATG CATTACAATA TGACGATAAA CTGCGCTTTA 380
ATGGAAAACC ATCCTATCGC TTTGAGCTTA AAGCCGAAGA CAATTCGCTT 430
GAAGGTTATG CTGCAGGAGA AACAAAGGGC CGTACAGAAT TGTCGTACAG 480
CTATGCAACC ACCAATGATT TTAAGAAATT TCCCCCAAGC GTATACCAAA 530
ATGCGCAAAA GCTAAAAACC GTTTATCATT ACGGCAAAGG GATTTGTGAA 580
CAGGGGAGCT CCCGCAGCTA TACCTTTTCA GTGTACATAC CCTCCTCCTT 630
CCCCGACAAT GCGACTACTA TTTTTGCCCA ATGGCATGGT GCACCCAGCA 680
GAACGCTTGT AGCTACACCA GAGGGAGAAA TTAAAACACT GAGCATAGAA 730
GAGTTTTTGG CCTTATACGA CCGCATGATC TTCAAAAAAA ATATCGCCCA 780
TGATAAAGTT GAAAAAAAAG ATAAGGACGG AAAAATTACT TATGTAGCCG 830
GAAAGCCAAA TGGCTGGAAG GTAGAACAAG GTGGTTATCC CACGCTGGCC 880
TTTGGTTTTT CTAAAGGGTA TTTTTACATC AAGGCAAACT CCGACCGGCA 930
GTGGCTTACC GACAAAGCCG ACCGTAACAA TGCCAATCCC GAGAATAGTG 980
AAGTAATGAA GCCCTATTCC TCGGAATACA AAACTTCAAC CATTGCCTAT 1030
AAAATGCCCT TTGCCCAGTT CCCTAAAGAT TGCTGGATTA CTTTTGATGT 1080
CGCCATAGAC TGGACGAAAT ATGGAAAAGA GGCCAATACA ATTTTGAAAC 1130
CCGGTAAGCT GGATGTGATG ATGACTTATA CCAAGAATAA GAAACCACAA 1180
AAAGCGCATA TCGTAAACCA GCAGGAAATC CTGATCGGAC GTAACGATGA 1230
CGATGGCTAT TACTTCAAAT TTGGAATTTA CAGGGTCGGT AACACCACGG 1280
TCCCGGTTAC TTATAACCTG AGCGGGTACA GCGAAACTGC CAGA.
1320

3. The nucleic acid molecule of claim 1 encoding the amino acid sequence (Sequence No. 2) consisting essentially of:

Met Lys Lys Gln Ile Leu Tyr Leu Ile Val Leu Gln Gln Leu Phe
1 5 10 15

```
Leu Cys Ser Ala Tyr Ala Gln Gln Lys Lys Ser Gly Asn Ile Pro
                20                  25                  30
Tyr Arg Val Asn Val Gln Ala Asp Ser Ala Lys Gln Lys Ala Ile
                35          40                  45
Ile Asp Asn Lys Trp Val Ala Val Gly Ile Asn Lys Pro Tyr Ala
                50                  55                  60
Leu Gln Tyr Asp Asp Lys Leu Arg Phe Asn Gly Lys Pro Ser Tyr
                65                  70                  75
Arg Phe Glu Leu Lys Ala Glu Asp Asn Ser Leu Glu Gly Tyr Ala
                80                  85                  90
Ala Gly Glu Thr Lys Gly Arg Thr Glu Leu Ser Tyr Ser Tyr Ala
                95                  100                 105
Thr Thr Asn Asp Phe Lys Lys Phe Pro Pro Ser Val Tyr Gln Asn
                110                 115                 120
Ala Gln Lys Leu Lys Thr Val Tyr His Tyr Gly Lys Gly Ile Cys
                125                 130                 135
Glu Gln Gly Ser Ser Arg Ser Tyr Thr Phe Ser Val Tyr Ile Pro
                140                 145                 150
Ser Ser Phe Pro Asp Asn Ala Thr Thr Ile Phe Ala Gln Trp His
                155                 160                 165
Gly Ala Pro Ser Arg Thr Leu Val Ala Thr Pro Glu Gly Glu Ile
                170                 175                 180
Lys Thr Leu Ser Ile Glu Glu Phe Leu Ala Leu Tyr Asp Arg Met
                185                 190                 195
Ile Phe Lys Lys Asn Ile Ala His Asp Lys Val Glu Lys Lys Asp
                200                 205                 210
Lys Asp Gly Lys Ile Thr Tyr Val Ala Gly Lys Pro Asn Gly Trp
                215                 220                 225
Lys Val Glu Gln Gly Gly Tyr Pro Thr Leu Ala Phe Gly Phe Ser
                230                 235                 240
Lys Gly Tyr Phe Tyr Ile Lys Ala Asn Ser Asp Arg Gln Trp Leu
                245                 250                 255
Thr Asp Lys Ala Asp Arg Asn Asn Ala Asn Pro Glu Asn Ser Glu
                260                 265                 270
Val Met Lys Pro Tyr Ser Ser Glu Tyr Lys Thr Ser Thr Ile Ala
                275                 280                 285
```

Tyr Lys Met Pro Phe Ala Gln Phe Pro Lys Asp Cys Trp Ile Thr
290 295 300
Phe Asp Val Ala Ile Asp Trp Thr Lys Tyr Gly Lys Glu Ala Asn
305 310 315
Thr Ile Leu Lys Pro Gly Lys Leu Asp Val Met Met Thr Tyr Thr
320 325 330
Lys Asn Lys Lys Pro Gln Lys Ala His Ile Val Asn Gln Gln Glu
335 340 345
Ile Leu Ile Gly Arg Asn Asp Asp Asp Gly Tyr Tyr Phe Lys Phe
350 355 360
Gly Ile Tyr Arg Val Gly Asn Ser Thr Val Pro Val Thr Tyr Asn
365 370 375
Leu Ser Gly Tyr Ser Glu Thr Ala Arg.
380

4. The nucleic acid molecule of claim 1 further comprising an expression vector.

5. The nucleic acid molecule of claim 1 further comprising a nucleic acid fragment encoding a signal peptide.

6. The nucleic acid molecule of claim 6 wherein the signal peptide is encoded by the nucleic acid (Sequence No. 1, base pairs 1 to 172, inclusive) consisting essentially of:

CCTTTTGGGA GCAAAGGCAG AACCATCTCC GAACAAAGGC AGAACCAGCC 50
TGTAAACAGA CAGCAATTCA TCCGCTTTCA ACCAAAGTGA AAGCATTTAA 100
TACAATACCA GAATGTCGCA TTTCCCTTTC AGCGTACTTT TTGGGTAAAT 150
AACCAATAAA AACTAAAGAC GA. 180

7. The nucleic acid molecule of claim 6 wherein the signal peptide directs the transport of the protein from the cytoplasm to the periplasm.

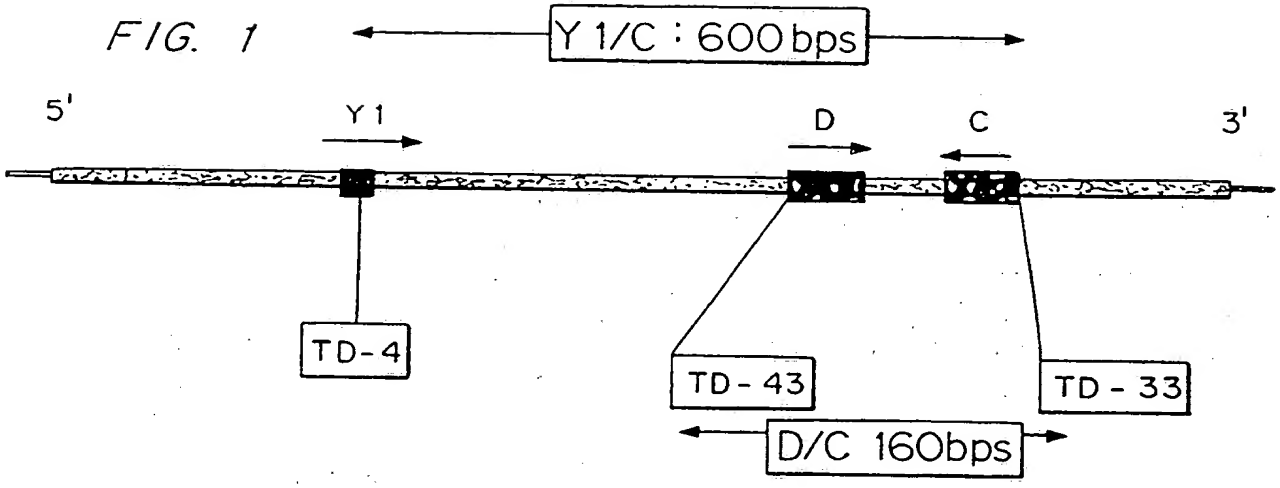
8. The nucleic acid molecule of claim 1 wherein the nucleic acid molecule encodes a heparinase having binding heparin with a different affinity than the heparinase encoded by Sequence No. 1.

9. The nucleic acid molecule of claim 1 wherein the nucleic acid molecule encodes a heparinase having a specific activity different from the specific activity of the heparinase encoded by Sequence No. 1.

10. The nucleic acid molecule of claim 1 in a procaryotic cell other than *F. heparinum* which is capable of expressing the molecule.

11. The nucleic acid molecule of claim 11 in a procaryotic cell cultured under low sulfate conditions which is capable of expressing the molecule.

FIG. 1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D
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3'
TD-4
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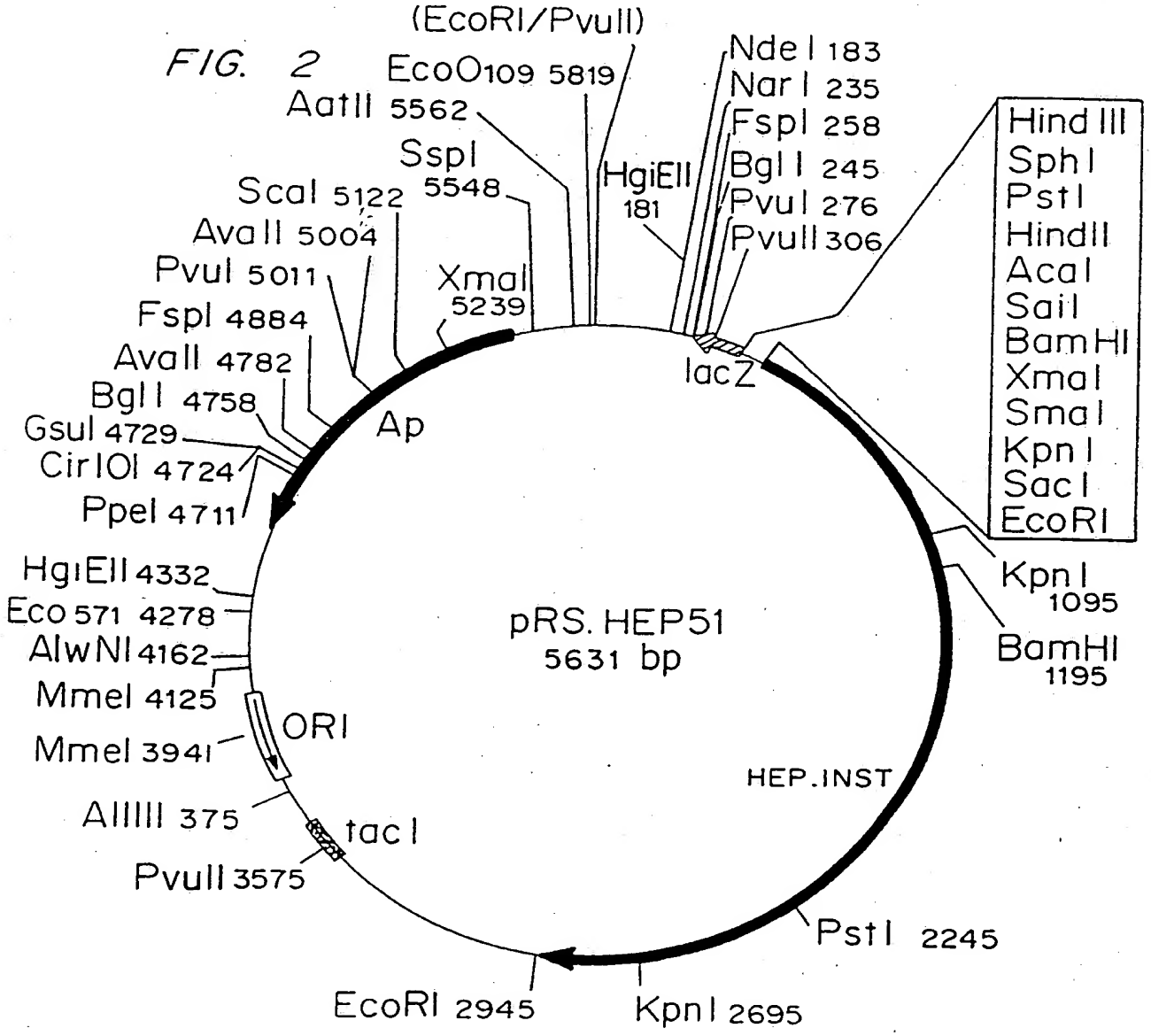
D/C 160bps

FIG. 2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EcoO109 5819
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PvuI 5011
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BglI 4758
GsuI 4729
CirIOI 4724
PpeI 4711
HgiEII 4332
Eco 571 4278
AlwNI 4162
MmeI 4125
MmeI 3941
AIIIII 375
PvuII 3575
EcoRI 2945
KpnI 2695
PstI 2245
HEP.INST
BamHI 1195
KpnI 1095
EcoRI
SacI
KpnI
SmaI
XmaI
BamHI
AcaI
HindII
PstI
SphI
HindIII
lacZ
Ap
ORI
tac I
pRS. HEP51
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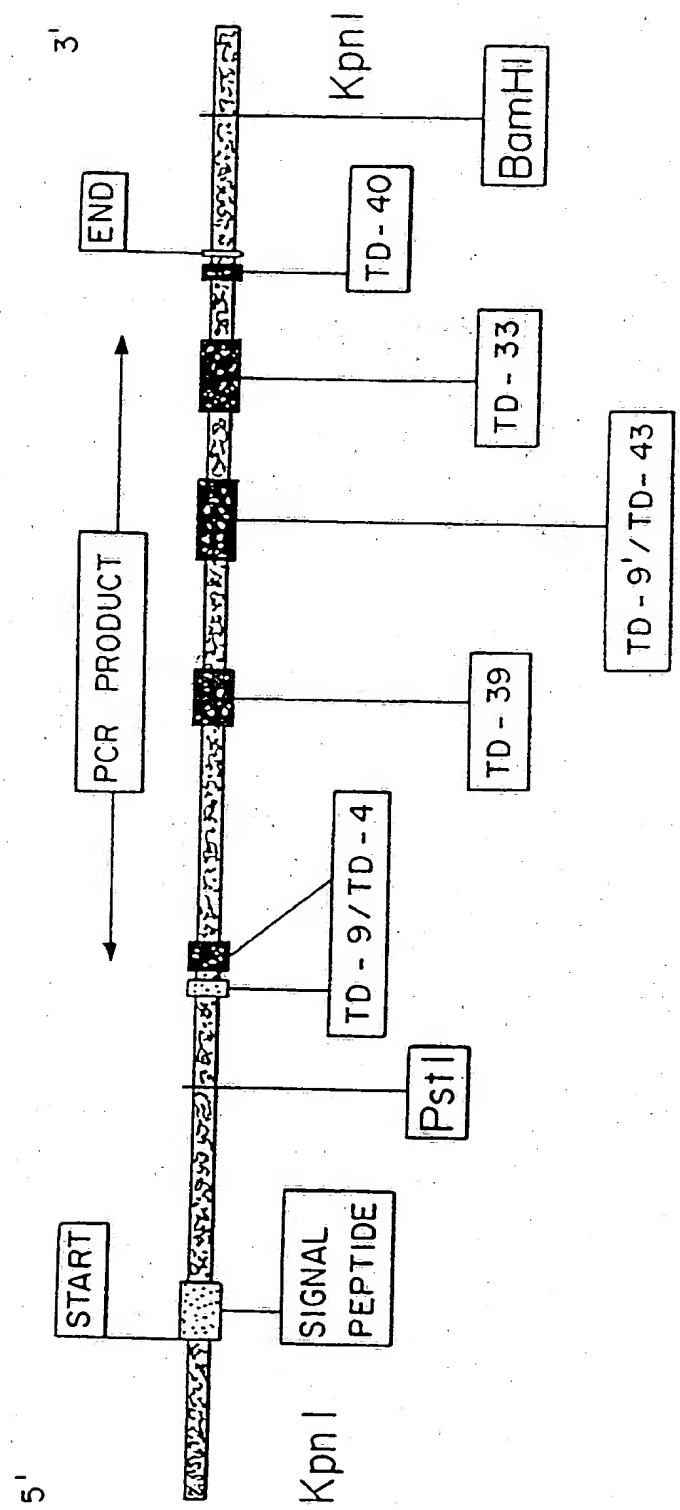
PvuI 306
BglI 245
FspI 258
NarI 235
NdeI 183

FIG. 3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TD-9/TD-4
PstI
START
SIGNAL PEPTIDE
Kpn I

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/09124

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/60

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO,A,8 912 692 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 28 December 1989 see page 18, line 30 - page 20, line 9 ---	1-11
X	Week 9125, Derwent Publications Ltd., London, GB; AN 91-180925 & JP,A,3 108 486 (SHINGIJUTSU KAIHATSU) 8 May 1991 see abstract -----	1-11

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 FEBRUARY 1993	09. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	CUPIDO M.

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9209124
SA 66558

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 19/02/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8912692	28-12-89	EP-A- 0420894	10-04-91
		JP-T- 3505815	19-12-91
		US-A- 5169772	08-12-92

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82